V. APPENDICES

A1. Staining protocol of Voltage FRET dyes

1. Reagents

(a) Assay buffer #1

140 mM NaCl
4.5 mM KCl
2 mM $CaCl_2$
1 mM $MgCl_2$
10 mM HEPES
10 mM glucose pH 7.40, 330 mOs/kg

(b) Pluronic stock (1000X)

100 mg/mL pluronic 127 in dry DMSO

(c) Oxonol stock (3333X)

10 mM $DiSBAC_2(3)$ in dry DMSO

(d) Coumarin stock (1000X)

10 mM CC2-DMPE in dry DMSO

(e) ESS-CY4 stock (400X)

200 mM ESS-CY4 in water

2. Loading and Assay Protocol

1. Preparation of CC2-DMPE loading buffer. Normally for a 96-well plate, 10 mL of staining solution will be prepared per plate.
   i) Mix equal volumes (10 µL) of coumarin stock and pluronic stock in a tube.
   ii) Add 10 mL Assay Buffer #1 to tube while gently vortexing.
   Loading concentration: 10 µM CC2-DMPE and 0.1 µg/ml pluronic.
2. Prepare oxonol loading buffer:
   i) Mix equal volumes (3.3 µL) of oxonol stock and pluronic stock in a tube.
   ii) Add 10 mL Assay Buffer #1 to tube while gently vortexing.
   iii) Add 25 µL ESS-CY4 while vortexing.
   Loading concentration: 3 µM $DiSBAC_2(3)$, 0.2 µg/ml pluronic, and 0.5 mM ESS-CY4.
   iv) If required, combine test compounds with the loading buffer at this time.
3. Rinse cells twice with Assay Buffer #1, removing all fluid from wells each time.
4. Add 100 µL CC2-DMPE loading buffer to each well. Incubate 30 minutes at room temperature, avoiding bright light.
5. Rinse cells twice with Assay Buffer #1, removing all fluid from wells each time.
6. Add 100 µL oxonol loading buffer to each well.

7. Incubate for 30 minutes at room temperature avoiding bright light. Use immediately.

A2. Analysis of VIPR™ reader data

Data were analyzed and reported as normalized ratios of intensities measured in the 460 nm and 580 nm channels. The process of calculating these ratios was performed as follows. On all plates, column 12 contained Assay Buffer #1 with the same DiSBAC2(3) and ESS-CY4 concentrations as used in the cell plates, however no cells were included in column 12. Intensity values at each wavelength were averaged in initial (before the stimulus) and final (during the stimulus) windows. These average values were subtracted from intensity values averaged over the same time periods in all assay wells. The ratios obtained from samples in the initial (Ri) and final windows (Rf) are defined as:

$$Ri = \frac{(\text{intensity 460 nm, initial} - \text{background 460 nm, initial})}{(\text{intensity 580 nm, initial} - \text{background 580 nm, initial})} \quad \text{(A2.1)}$$

$$Rf = \frac{(\text{intensity 460 nm, final} - \text{background 460 nm, final})}{(\text{intensity 580 nm, final} - \text{background 580 nm, final})} \quad \text{(A2.2)}$$

Final data are normalized to the starting ratio of each well and reported as Rf/Ri.

A3. Screening Window

The screening window W for a response is defined as follows. Data from multiple wells at identical stimulus conditions are required. The control wells can either be pharmacologically blocked or untransfected cell stimulated with the full electric field. Alternatively, one might use transfected cells with no stimulus applied.

Responses from experimental and control wells are measured. The average and standard deviations of the responses in the experimental ($R \pm \Delta R$) and control ($C \pm \Delta C$) wells are calculated. The screening window is defined as the difference between experimental and control signals normalized to the sum of the standard deviations.

$$W = \frac{R - C}{\Delta R + \Delta C} \quad \text{(A3.1)}$$

A general rule of thumb for an acceptable screening window is $W>3$. This allows one to choose a cutoff line midway between control and experimental responses which ensures a false negative/positive rate less than 1%. Assuming a normal distribution, the false positive/negative rate as a function of the screening window W is:

$$P_{false} = 1 - prob(W) = 1 - \frac{1}{\sqrt{2\pi}} \int_{-W}^{+W} \exp\left(-\frac{t^2}{2}\right) dt \quad \text{(A3.2)}$$

Table A3.1. The false positive/negative rate *P(W)* as a function of the screening window *W* as defined in Equation A3.1. This calculation assumes that the cutoff for identification of a hit is placed an equal number of standard deviations away from the positive and negative control responses.

W	P(W)
1	0.3173
2	0.0455
3	0.0027
4	6.334E-5
5	5.733E-7
6	1.973E-9
7	2.559E-12
8	1.221E-15
9	<1E-18
10	<1E-18